New Mutant *N*-Acetylglutamate Synthase and Method for L-Arginine Production

Background of the Invention

Field of the Invention

The present invention relates to microbiological industry, to the method of L-arginine production and concerns the using of new feedback-resistant mutant enzymes in arginine biosynthesis pathway of *E. coli* arginine-producer strains.

Description of the Related Art

The biosynthesis of arginine from glutamate in *E. coli* cells is carried out by a series of reactions initiated by the acetylation of glutamate by *N*-acetylglutamate synthase (NAGS) encoded by *argA*. This process is regulated via transcription repression of the *arg* regulon and by feedback inhibition of NAGS by arginine [Cunin R., *et al.*, *Microbiol. Rev.*, *vol.50*, p.314-352, 1986]. L-Arginine represses *argA* expression with a ratio greater than 250 and inhibits NAGS activity (*Ki* = 0.02 mM) [Leisinger T., Haas D., *J. Biol. Chem.*, *vol.250*, p.1690-1693, 1975]. For enhanced biosynthesis of arginine in *E. coli*, the feedback-resistant (may be referred to as "fbr") NAGS enzymes are required.

The feedback-resistant mutants of enzymes can be

obtained by spontaneous, chemical or site-directed mutagenesis.

Some *argA* fbr mutants were isolated and studied. The *Serratia marcescens* cells carrying the chromosomal fbr *argA* mutations were unstable and gave rise to *argA* mutants with reduced activity or with altered affinity for glutamate [Takagi T., *et al.*, *J.Biochem. vol.99*, p.357-364 1986].

The fbr *arg*A genes from the five *E. coli* strains with fbr NAGS were cloned and different single-base substitutions in *argA* genes were found in each of the fbr NAGS strains and it was revealed that the substitutions cause replacing His-15 with Tyr, Tyr-19 with Cys, Ser-54 with Asn, Arg-58 with His, Gly-287 with Ser and Gln-432 with Arg (Rajagopal B.S. *et al.*, *Appl. Environ. Microbiol.*, 1998, *vol.64*, No.5, p. 1805-1811).

As a rule, the fbr phenotype of enzyme arises as a result of the replacing the amino acid residue with another in a single or in a few sites of protein sequence and these replacements lead to reducing the activity of enzyme. For example, the replacing of natural Met-256 with 19 other amino acid residues in *E. coli* serine acetyltransferase (SAT) (*cysE* gene) leads in most cases to fbr phenotype but the mutant SAT proteins do not restore the level of activity of natural SAT (Nakamori S. *et al.*, *AEM*, *64*(5):1607-11, 1998).

So, the disadvantage of the mutant enzymes,

obtained by these methods, is a reduce in the activity of mutant enzymes as compared to wild type enzymes.

Summary of the Invention

An object of the present invention is to provide mutant feedback resistant and high active enzymes which play a key role in biosynthesis of arginine by *E. coli*.

In present invention the novel procedure for synthesis a large set of mutant *argA* genes is proposed by using the full randomization of fragment of *argA* gene. The simultaneous substitutions of some amino acid residues in fragment of protein sequence, in which the fbr mutation can be localized, can be able to give a mutant proteins with restored the level of its activity near to natural due to more correct restored three dimensional structure of enzyme. Thus the present invention described below has been accomplished.

That is the present invention provides:

(1) A mutant N-acetylglutamate synthase wherein the amino acid sequence corresponding to positions from 15 to 19 in a wild type N-acetylglutamate synthase is replaced with any one of amino acid sequences of SEQ ID NOS: 1 to 4, and feedback inhibition by L-arginine is desensitized;

(2) The mutant N-acetylglutamate synthase according to (1), wherein a wild type N-acetylglutamate synthase is

that of *Escherichia coli*.

(3) The mutant N-acetylglutamate synthase according to (1), which includes deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions other than positions from 15 to 19, wherein feedback inhibition by L-arginine is desensitized;

(4) A DNA coding for the mutant N-acetylglutamate synthase as defined in any one of (1) to (3);

(5) A bacterium belonging to the genus *Escherichia* which is transformed with the DNA as defined in (4) and has an activity to produce L-arginine; and

(6) A method for producing L-arginine comprising the steps of cultivating the bacterium as defined in (5) in a medium to produce and accumulate L-arginine in the medium and collecting L-arginine from the medium.

The NAGS having any of fbr mutation as described above may be referred to as "the mutant NAGS", a DNA coding for the mutant NAGS may be referred to as "the mutant *argA* gene", and a NAGS without mutation may be referred to as "a wild type NAGS".

Hereafter, the present invention will be explained in detail.

<1> Mutant NAGSs and mutant *argA* genes

The mutant NAGSs and the mutant *argA* genes coding the same were obtained by randomized fragment-directed

mutagenesis. To obtain the numerous mutations in *argA* gene the full randomization of 15-nucleotide fragment of *argA* gene which codes the region from 15-th to 19-th amino acid residues in protein sequence was carried out. The full randomized 15-nucleotide fragment gives 4^{15} or near 10^9 different DNA sequences which can code 20^5 different amino acid residues in 5-mer peptide. The likelihood of in frame non-introducing the *stop* codons in this sequences is equal of about 0.95^5 or 78%. So, the full randomization of the *argA* gene fragment coded the peptide from 15-th to 19-th amino acid residues must give approximately 2.5 million different protein sequences with diversity in this peptide fragment of NAGS structure. Subsequent selection and screening of recombinant clones carrying mutant *argA* genes cloned into expression vector allows to choose the fbr variants of mutant NAGS with different level of its biological activity up to level of activity of derepressed wild-type (wt) NAGS. In the selection, the inventors considered that the strain harboring the mutant *argA* gene would be obtained by using *argD*$^-$, and *proB*$^-$ or *proA*$^-$ strain, because such a strain cannot produce L-proline due to inhibition of NAGS thereby cannot grow if excess amount of L-arginine exists in a culture medium, but the strain harboring fbr NAGS can grow in a minimal medium because glutamate-semialdehyde, a precursor of L-proline, can be supplied by acetylornithine deacetylase (the argE

product) from N-acetylglutamate-semialdehyde, a precursor of L-arginine (Eckhardt T., Leisinger T., *Mol. Gen. Genet., vol. 138*, p.225-232, 1975). However, the inventors found that it is difficult to obtain fbr NAGS having high activity by the above method as described in the abter-mentioned following Example, and that fbr NAGS having high activity can be obtained by introducing the mutant argA into a wild type strain and selection of a strain which shows delay of cell growth.

The amino acid sequences of the mutant NAGS suitable for fbr phenotype of NAGS were defined by the present invention. Therefore, the mutant NAGS can be obtained based on the sequences by introducing mutations into a wild type *argA* using ordinary methods. As a wild type *arg*A gene, the *argA* gene of *E. coli* can be mentioned (GenBank Accession Y00492).

The amino acid sequence of positions from 15 to 19 in the mutant NAGS of the present invention is any one of the sequnece of SEQ ID NOS: 1 to 4. The corresponding amino acid sequence of known mutant NAGS, in which tyr at a position 19 is replaced with Cys, and the wild type NAGS of *E. coli* are illustrated in SEQ ID NOS: 5 and 6. Examples of nucleotide sequence encoding these amino acid sequences are shown in SEQ ID NOS: 7 to 12. Table 1 shows these sequence.

Table 1

Amino acid sequence	SEQ ID NO:	Nucleotide sequence	SEQ ID NO:
Val Val Trp Arg Ala	1	GTAGTATGGCGGGCA	7
Leu Phe Gly Leu His	2	TTGTTCGGATTGCAC	8
Ser Arg Arg Ser Arg	3	TCGCGGCGGTCCAGA	9
Gly Trp Pro Cys Val	4	GGGTGGCCATGCGTG	10
His Ser Val Pro Cys	5	CATTCGGTTCCCTGT	11
His Ser Val Pro Tyr	6	CATTCGGTTCCCTAT	12

The mutant NAGS may including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions other than 15th to 19th, provided that the NAGS activity, that is an activity to catalyze the reaction of acetylation of L-glutamic acid which produces N-acetylglutamate, is not deteriorated.

The number of "several" amino acids differs depending on the position or the type of amino acid residues in the three dimensional structure of the protein. This is because of the following reason. That is, some amino acids have high homology to one another and the difference in such an amino acid does not greatly affect the three dimensional structure of the protein. Therefore, the mutant NAGS of the present invention may be one which has homology of not less than 30 to 50 %, preferably 50 to 70 % with respect to the entire amino acid residues for constituting NAGS, and which has the fbr NAGS activity.

In the present invention, "amino acid sequence correspoding to the sequence of positions from 15 to 19"

means an amino acid sequence corresponding to the amino acid sequence of positions from 15 to 19 in the amino acid sequence of *E. coli* wild type NAGS. A position of amino acid residue may change. For example, if an amino acid residue is inserted at N-terminus portion, the amino acid residue inherently locates at the position 15 becomes position 16. In such a case, the amino acid residue corresponding to the original position 15 is designated as the amino acid residue at the position 15 in the present invention.

The DNA, which codes for the substantially same protein as the mutant NAGS as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site involve deletion, substitution, insertion, or addition. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating a DNA containing the mutant *argA* gene in vitro, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium, belonging to the genus *Escherichia* harboring the mutant *argA* gene with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoquanidine (NTG) and nitrous acid usually used for the mutation treatment.

The substitution, deletion, insertion, or addition of nucleotide as described above also includes mutation which naturally occurs (mutant or variant), for example, on the basis of the individual difference or the difference in species or genus of bacterium which harbors NAGS.

The DNA, which codes for substantially the same protein as the mutant *argA* gene, is obtained by expressing DNA having mutation as described above in an appropriate cell, and investigating NAGS activity of an expressed product.

Also, the DNA, which codes for substantially the same protein as the mutant NAGS, can be obtained by isolating a DNA which hybridizes with DNA having known *argA* gene sequence or a probe obtainable therefrom under stringent conditions, and which codes for a protein having the NAGS activity, from a cell harboring the mutant NAGS which is subjected to mutation treatment.

The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 50% with each other are hybridized, and DNAs having homology lower than the above with each other are

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not hybridized. Alternatively, the stringent condition is exemplified by a condition under which DNA's are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 60°C, preferably 65°C, 1 x SSC, 0.1 % SDS, preferably 0.1 x SSC, 0.1 % SDS.

The gene, which is hybridizable under the condition as described above, includes those having a stop codon generated within a coding region of the gene, and those having no activity due to mutation of active center. However, such inconveniences can be easily removed by ligating the gene with a commercially available expression vector, and investigating NAGS activity.

<2> Bacterium belonging to the genus *Escherichia* of the present invention

The bacterium belonging the genus *Escherichia* of the present invention is a bacterium belonging to the genus *Escherichia* to which the mutant *argA* gene as described above is introduced. A bacterium belonging to the genus *Escherichia* is exemplified by *E. coli*. The mutant *argA* gene can be introduced by, for example, transformation of a bacterium belonging to the genus *Escherichia* with a recombinant DNA comprising a vector which functions in a bacterium belonging to the genus Escherihia and the mutant *argA* gene. The mutant *argA*

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gene can be also introduced by substitution of *argA* gene on a chromosome with the mutant *argA* gene.

Vector using for introduction of the mutant *argA* gene is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, phage vectors such as l1059, lBF101, M13mp9 or the like and transposon such as Mu, Tn10, Tn5 or the like.

The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. A. Morrison (Methods in Enzymology, 68, 326 (1979)) or a method in which recipient bacterial cell are treated with calcium chloride to increase permeability of DNA (Mandel, M., and Higa, A., *J. Mol. Biol.*, *53*, 159, (1970)) and the like.

If the mutant *argA* gene is introduced into L-arginine-producing bacterium belonging to the genus *Escherichia* as described above, a produced amount of L-arginine can be increased. Besides, an ability to produce L-arginine may be imparted to a bacterium to which the mutant *argA* gene is introduced.

As the bacterium belonging to the genus *Escherichia* which has an activity to produce L-arginine is exemplified by *E. coli* 237 strain (VKPM B-7925). The 237 strain has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) under the accession number VKPM B-7925 since April 10, 2000, and

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transferred to the original deposit to international deposit based on Budapest Treaty, on May 18, 2001.

<3> Method for producing L-arginine

L-arginine can be efficiently produced by cultivating the bacterium to which the mutant *argA* gene is introduced and which has an ability to produce L-arginine, in a culture medium, producing and accumulating L-arginine in the medium, and collecting L-arginine from the medium.

In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of L-arginine from the liquid medium may be performed in a manner similar to those of the conventional method for producing L-arginine by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amount. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids, depending on assimilatory ability of the used bacterium. Alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone,

soybean hydrolyzate and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

The cultivation is preferably culture under an aerobic condition such as a shaking, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the accumulation of L-arginine in the medium.

Collecting L-arginine can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying L-arginine by ion exchange, concentration and crystalline fraction methods and the like.

Brief explanation of the Drawings

Fig. 1 shows scheme of construction of pool of mutant *argA* genes.

Best Mode for Carrying out the Invention

The present invention will be specifically explained with reference to the following examples.

Example 1

<1> The randomized fragment-directed mutagenesis

The *Bam*HI-*Sal*I chromosomal DNA fragment (2.02 kb) with *wt argA* gene was cloned into plasmid pUC19 (plasmid pUC19-ArgA). Pyrobest™ DNA Polymerase used for PCR amplification was obtained from Takara Shuzo Co. (Japan) and is used under the conditions recommended by the supplier.

To construct the pool of mutant *argA* genes, at the first step the fragment of *argA* gene coded the sequence from 20-th amino acid residue to the end of NAGS was amplifying (Fig.1). The plasmid pUC19-ArgA is used as the template, the sense primer P1: 5'-CGAGGGATTCCGCNNNNNNNNNNNNNNNATCAATACCCACCGGG-3'(SEQ ID NO:13), is designed based on the nucleotide sequence of *argA* and the standard M13 reverse sequence primer is used as a antisense primer P2. The fixed 16-nucleotide 3'-end sequence of primer P1 is homologous to the sequence of *argA* gene downstream Tyr-19 codon and the fixed 13'-nucleotide 5'-end - to the sequence upstream His-15. The homology of 3'-end part of P1 to *argA* sequence was used to synthesize the 1.75 kbp DNA fragment by using twenty PCR cycles.

100ng of pUC19-ArgA was added as a template to PCR solution (50 μl) containing each of the two primers (40 pmol). Twenty PCR cycles (94°C for 0.6 min, 55°C for 0.5 min, 72°C for 2 min) is carrying out with a model 2400 DNA thermal cycler (Perkin-Elmer Co., Foster City, Calif.)

At the second step of amplification eight cycles (94°C for 1 min, 37°C for 1 min, 72°C for 0.5 min) is carrying out in which the (-) chain of this fragment is functioning as a "primer" for extension it to get the full gene sequence.

At the third step, the 10 μl aliquot of the reaction mixture is added to a fresh reaction mixture (40 μl) containing 100 pmol of the sense primer P3: 5'-TGCCATGGTAAAGGAACGTAAAACC-3'(SEQ ID NO:14), homologous to 5'-end sequence of *argA* gene, and primer P2 as antisense, and additional ten cycles (94°C for 0.5 min, 52°C for 0.5 min, 72°C for 2 min) are performed.

The 1.78 kbp DNA fragment coding the pool of mutant variants of full length *argA* genes is purified by agarose gel electrophoresis, is digested with *Nco*I (the site which includes the initial ATG codon of *argA* gene) and *Hin*dIII, and then is ligated to the pKK233-2 vector (Pharmacia, Sweden) digested with *Nco*I and *Hin*dIII.

About 150 ng of DNA ligated is used for transformation of *E. coli* recipient cells in subsequent

experiments to give about 2000 recombinant clones in each case.

<2> Isolation of new *argA* mutants and effect of amino acid substitutions in NAGS on catalytic properties

The plasmid vector pKK 233-2 (Pharmacia, Sweden) was used for cloning and expression of *argA* gene variants. The *E. coli* recipient strain was TG1 (*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) (J.Sambrook et al., *Molecular Cloning*, 1989). The selection TG1 cells carrying the set of recombinant plasmids pKK-argA-random (with *argA* gene mutants) was carried out on LB agar plates. The delay of cell growth of some mutant clones was observed and this effect was supposed to correlate with production of the active fbr NAGS mutant enzymes. The plasmids from some clones was purified and DNA sequence of 5'-fragments of mutant *argA* genes was determined by using dideoxy chain termination method (table 2).

To determine the NAGS activity, the arginine auxotroph, strain *E. coli* B3083 (*argA*$^-$, *metB*$^-$) is transformed with these plasmids. The enzymes in the soluble fractions obtained from sonicated recombinant cells is partly purified by ammonium sulfate precipitation and assayed as described below. The NAGS activity of strains carrying plasmids pKK-argA-r11 (3390 nmol/min x mg), pKK-argA-r12 (1220 nmol/min x mg) and

pKK-argA-r13 (3120 nmol/min x m g) is significantly higher than the NAGS activity of the strain harboring pKK-argA-r4 (300 nmol/min x mg). The last plasmid carried the mutant *argA* gene with the same substitution (Y19C) as it was described for most active variant of *argA* gene with single substitution by Rajagopal B.S. *et al.* (Rajagopal B.S. *et al.*, *Appl. Environ. Microbiol.*, 1998, v.*64*, No.5, p. 1805-1811).

Also, the activity of NAGS in strain carrying the plasmid pKK-argA(wt) (wild type *argA*) is lower than in the case of pKK-argA-r11, -r12 and -r13. The levels of activity of mutant enzymes are approximately the same in presence of 10 mM arginine, while the wild-type enzyme is markedly inhibited by arginine (less than one-tenth). These results indicate that peptide fragment from 15-th to 19-th amino acid residues is responsible for the feedback inhibition of NAGS by L-arginine and for the level of catalytic efficiency of mutant NAGS.

(Enzyme assay)

The acetyl coenzyme A and all chemicals used were purchased from Sigma Chemical Co., St. Louis, Mo. To determinate NAGS activities, cells *E. coli* B3083 (*argA*$^-$, *metB*$^-$) carrying recombinant plasmids are grown in M9 medium (5 ml) to the late exponential phase, washed with 0.14 M NaCl solution, and resuspended in 2 ml of 40 mM K-phosphate buffer (pH 7.0) with 100 mM KCl.

The cells is sonicated and centrifuged. The NAGS containing fractions are precipitated by 5 volumes of saturated $(NH_4)_2SO_4$ and pellets are dissolved in 2 ml of 40 mM K-phosphate buffer (pH 7.0) with 100 mM KCl and 30% (vol/vol) glycerol. The NAGS solution is added to 0.1 ml of reaction mixture (100 mM tris-HCl (pH 8.5), 35 mM KCl, 20 mM L-glutamate, 1.2 mM acetyl coenzyme A) and reaction mixture is incubated at 37°C for 10 min. The reaction is stopped by adding 0.3 ml of ethanol and reaction mixture is centrifuged. 0.95 ml of 0.24 mM DTNB (5,5-dithio-bis-2-nitrobenzoate) solution is added to supernatant and mixture is incubated for 15 min. The NAGS activity is assayed by measuaring the absorbance at 412 nm.

<3> Isolation of new *argA* mutants by selection in B16-4 (*pro*$^-$ *argD*$^-$) cells

The selection of mutant *argA* (pKK-argA-random) in *E. coli* B16-4 strain (*pro*$^-$ *argD*$^-$) was carried out by the above described procedure. The recombinant clones from agar plates were suspended in M9 medium with L-arginine, and were grown to stationary phase. The aliquot of culture was suspended in the fresh medium and the growth procedure was repeated four times. After that aliquot of culture is plated on M9 agar with 5 mg/ml of L-arginine and 100 μg of ampicillin. The plasmids from some clones were purified, 5'-fragments of mutant *argA*

genes were sequenced and the levels of activity of mutant NAGS were assayed as described above. The 60% of mutants carried the sequence -Gly-Trp-Pro-Cys-Val- (SEQ ID NO: 4) in a mutagenized fragment of enzyme and possessed a weak (about 10 nmol/min x mg) but fbr NAGS activity. Obviously, this mutant protein provides the optimal level of NAGS activity for the growth of *pro*⁻ *argD*- cells in the selection conditions used. So, the conditions of selection are supposed to determine the activity of the mutant NAGS obtained.

Table 2 NAGS(ArgA) obtained by randomized fragment-directed mutagenesis

Clone with Recombinant	Sequence of altered fragment of mutant *argA* gene	Altered sequence of NAGS (fragment of protein from 15-th to 19-th a.a.)	NAGS activity, nmol/min x mg*	NAGS activity in the presence of L-Arg (10 mM), %**
PKKArgA-r11	GTAGTATGGCGGGCA	ValValtrpArgAla	3390	103%
PKKArgA-r12	TTGTTCGGATTGCAC	LeuPheGlyLeuHis	1220	100%
PKKArgA-r13	TCGCGGCGGTCCAGA	SerArgArgSerArg	3120	107%
PKKArgA-32-34,36,38.39	GGGTGGCCATGCGTG	GlyTrpProCysVal	10.3	103%
PKKArgA-r4	CATTCGGTTCCCTGT	HisSerValProCys	300	91%
PKKArgA-wt	CATTCGGTTCCCTAT	HisSerValProTyr	1200	<10%

* To total cellular proteins;

** 100% stands for activity in the absence of L-Arg.

<4> Production of L-arginine by using of mutant *argA* genes

The recombinant plasmids pKKArgA-r4, 11, 12, 13 and 32 were digested by *Bam*HI and *Sal*I, and the fragments which contained mutant *argA* genes under *trc* promoter were sub-cloned onto low copy plasmid pMW119 (Nippon Gene Co., Tokyo). Resulting plasmids were

designated pMADS4, pMADS11, pMADS12, pMADS13 and pMADS32, respectively. These plasmids were introduced into an L-arginine-producing strain *E. coli* 237 (VKPM B-7925). The L-arginine (Arg) and citrulline (Cit) production of transformants are shown in Table 3. Most of the producer strains with the new mutant NAGS'es give the higher Arg+Cit production than the recipient strain or strain with known Tyr19Cys mutant NAGS (pMADS4).

Table 3. Production of L-arginine and citrulline.

Strain	Arg (g/l)	Cit (g/l)	Arg + Cit (g/l)
237	4.7	0	4.7
237/pMADS4	8.7	0	8.7
237/pMADS11	10.0	3.0	13.0
237/pMADS12	7.6	2.6	10.2
237/pMADS13	9.1	3.7	12.8
237/pMADS32	8.2	0	8.2

(The cultivation conditions in test-tube fermentation)

The fermentation medium contained 60 g/l glucose, 25 g/l ammonia sulfate, 2 g/l KH_2PO_4, 1 g/l $MgSO_4$, 0.1 mg/l thiamine, 5 g/l yeast extract Difco, 25 g/l chalk, per 1 liter of tap water (pH 7.2). Glucose and chalk were sterilized separately. 2 ml of the medium was placed into test-tubes, inoculated with one loop of the tested microorganisms, and the cultivation was carried out at 32° C for 3 days with shaking.